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An optical method for reducing green fluorescence from urine during fluorescence-guided cystoscopy

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Abstract: Photodynamic diagnosis (PDD) of bladder tumour tissue significantly improves endoscopic diagnosis and treatment of bladder cancer in rigid cystoscopes in the operating theatre and thus reduces tumour recurrence. PDD comprises the use of blue light, which unfortunately excites green fluorescence from urine. As this green fluorescence confounds the desired red fluorescence of the PDD, methods for avoiding this situation particularly in cystoscopy using flexible cystoscopes are desirable. In this paper we demonstrate how a tailor made high power LED light source at 525 nm can be used for fluorescence assisted tumour detection using both a flexible and rigid cystoscope used in the outpatient department (OPD) and operating room (OR) respectively. It is demonstrated both *in vitro* and *in vivo* how this light source can significantly reduce the green fluorescence problem with urine. At the same time this light source also is useful for exciting autofluorescence in healthy bladder mucosa. This autofluorescence then provides a contrast to the sensitized fluorescence (PDD) of tumours in the bladder.

Keywords: (170.2150) Endoscopic imaging; (170.2655) Functional monitoring and imaging; (170.6280) Spectroscopy fluorescence and luminescence; (170.7230) Urology; (230.3670) Light emitting diodes; (350.5130) Photochemistry

1. Introduction

According to a recently published review article on bladder cancer (Antoni *et al* 2016), this cancer form accounted for almost 430.000 new cases diagnosed in 2012 worldwide. A further look into the numbers related to bladder cancer found on the homepage of the International Agency for Research on Cancer reveals that more men than women are affected by this disease. The numbers worldwide are found in table 1 below.

Table 1. Worldwide incidence, mortality and 5 year prevalence of bladder cancer in men and women according to GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide (http://globocan.iarc.fr/Pages/fact_sheets_population.aspx).

	Men	Women
Incidence	330,380	99,413
Mortality	123,051	42,033
5 Year prevalence	1,018,415	301,334

One of the most common surgical procedures for eradicating bladder tumours is transurethral resection of bladder tumours (TUR-B). This is an endoscopic procedure recommended in the European guidelines for treatment of bladder cancer (Babjuk *et al* 2011). Fluorescence guided cystoscopy of the bladder is an integral part of TUR-B for identifying tumours in the bladder but also as a subsequent follow-up procedure (Hermann *et al* 2011). The fluorescence guided cystoscopy, also known as photodynamic diagnosis (PDD), is based on administration of an aqueous solution of an amino acid, hexylaminolevulinic acid (HAL), to the bladder through a catheter inserted in the urethra of the patient prior to TUR-B. The tumour cells convert this amino acid into a fluorescent substance Protoporphyrin IX (PpIX) (Krieg *et al* 2000). This dye has a characteristic red fluorescence centred at 635 nm which differs spectrally from the autofluorescence of the healthy bladder tissue (Bulgakova *et al* 2009). The advantage of PDD over conventional white-light cystoscopy (WLC) is its higher specificity, particularly towards low-grade tumours (Kausch *et al* 2010). HAL is preferred over 5-aminolevulinic acid (5-ALA) due to its higher uptake by bladder tumour cells (Jocham *et al* 2008).

To provide the surgeon with suitable excitation light, a light source with two illumination modes is used; blue light 430 nm (50 nm FWHM) and white light (400 nm to 700 nm). The blue light mode is intended to reveal the location of the bladder tumours through excitation of a strong red fluorescence, whereas the white light is used for general observation of the bladder mucosa by the surgeon. The most predominant type of light sources used in the clinic are Karl Storz (D-Light) and Olympus EXERA II CLV 180.

However, urine contains fluorescent metabolites, typically riboflavines (Lovisa *et al* 2010, Leiner *et al* 1987), which are excited to emit green fluorescence by the blue light used in PDD. The green fluorescence, as shown in figure 1, from these metabolites makes it impossible for the urologist to see details in the bladder. This impairs the use of PDD with flexible cystoscopes in the outpatient department (OPD). The green colour problem is limited in the operating room (OR) because green fluorescent metabolites from the urine are diluted by continuous bladder irrigation used in the OR. Bladder irrigation is possible in the thick rigid cystoscopes used in the OR setting, but limited in the small flexible endoscopes used in the OPD.

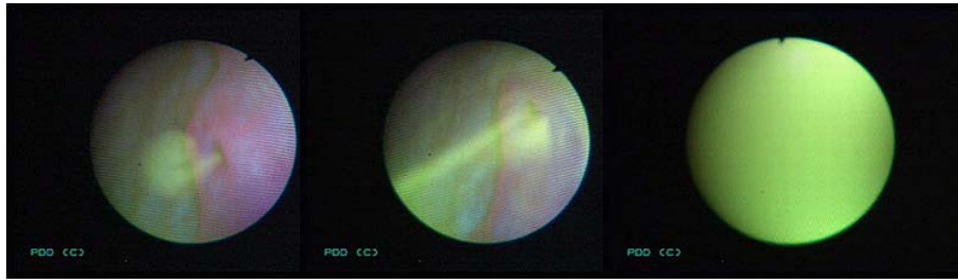


Figure 1. Three images of green fluorescence from urine excited by blue light (430 nm) observed in a flexible cystoscope in the OPD, showing the increased obscuration of the view of the bladder wall as the urine enters the bladder from the kidney over an interval of 0, 5 and 10 seconds. © Gregers G. Hermann.

A previous study (Hermann *et al* 2012) has shown that histological diagnosis of non-muscle invasive bladder cancer (NMIBC) in biopsies obtained during flexible cystoscopy in the OPD in local anaesthesia, is as good as biopsies obtained through rigid cystoscopes the OR from patients in general anaesthesia (which is commonly regarded as the ‘gold standard’ for this purpose). There is therefore an impetus to move approximately 20% of patients with NMIBC from the OR to the OPD, which is faster, less resource-intensive, and works better for the patient and the healthcare system.

In this paper we describe the *in vivo* use of a high-power LED light source for PDD developed at our laboratory, which reduces the green fluorescence of urine significantly. Using this light source, only one wavelength (525nm) is required to excite the red fluorescence of tumours and concomitant autofluorescence of the bladder. In conventional PDD, the surgeon has to switch between blue and white light to perform diagnosis and surgery respectively. The procedures are carried out with rigid cystoscope in the operating room (OR) and with a flexible cystoscope in the outpatient department (OPD). The proof of principle presented in this paper was done using commercially available cystoscopes and light guides. Due to the delicate nature of inserting surgical instruments, e.g., a cystoscope, into the human bladder, only certified equipment can be used. Furthermore, the use of commercially available instruments allows us to follow the European guidelines (Babjuk *et al* 2011) for transurethral resection of bladder tumours (TUR-B).

2. Materials and Methods

2.1 Preparation of Protoporphyrin IX (PpIX) for fluorescence measurements *in vitro*

60.6 mg of Protoporphyrin IX disodium salt (equivalent of 0.100 mmol) were dissolved in a phosphate buffered saline solution (PBS) to make a total volume of 100 ml in a volumetric flask containing 1.00 ml Triton X-100 as surfactant to prevent agglomeration of the PpIX molecules, making up a 1.00 mM solution. This solution was further diluted by PBS containing 1% Triton-X to a 100 μ M solution for measuring the absorption and fluorescence spectra respectively. The Protoporphyrin IX disodium salt was supplied by Sigma Aldrich# 258385, (Sigma-Aldrich Denmark A/S, Vallensbæk, Denmark). Triton X-100 used was

Sigma Aldrich# X-100, (Sigma-Aldrich Denmark A/S, Vallensbæk, Denmark). Phosphate buffered saline (PBS) solution was made by dissolving a package of pre-mixed buffer from Sigma-Aldrich# P5368 water (Sigma-Aldrich Denmark A/S, Vallensbæk, Denmark) in a 1000 ml volumetric flask using MilliQ water). The pH of the PBS was 7.4.

2.2 Administration of Protoporphyrin IX in vivo

In fluorescence-guided cystoscopy of the bladder, also known as photodynamic diagnosis (PDD), a 50 ml solution of a photosensitiser precursor, the hexyl derivative of 5-aminolevulinic acid (Hexvix[®], Photocure ASA, Oslo, Norway), is administered to the bladder through a catheter via the urethra. This substance is metabolised in the mitochondria of the tumour cells to Protoporphyrin IX (PpIX) as a part of the heme cycle (Hamblin and Mróz). PpIX emits red fluorescence (635 nm), making the tumours of the bladder fluoresce red when the bladder is illuminated by blue light (430 nm) through the cystoscope.

2.3 The LED light source

To match the optical power required for the cystoscope, a tailor-made LED-based light source has been developed in our laboratory. Only the light source is tailor-made but with a light guide connection identical to that used in fluorescence assisted cystoscopy (PDD). Consequently, a user familiar with conventional photodynamic diagnosis of bladder tumours will immediately be able to use the new light source

A detailed description of this light source can be found in a previous publication (Lindvold and Hermann 2015). In short, this light source is based on high power Luminus Devices PT-121-TE with a centre wavelength of 525 nm. Connected to a Storz 495NCS light guide, an optical output power density of 1.6 W/cm² can be obtained before it is coupled to the cystoscope optics. It should be noted that the high power density is required to compensate for the insertion loss of almost 90% between the output of the light guide and the cystoscope optics. The spectral bandwidth of the light source is limited by a Semrock Brightline 525-25 bandpass filter. The bandpass filter serves two purposes, as shown in figure 2. One is to cut off any blue light from the LED that could excite green fluorescence from the urine and cause bleaching of the photosensitiser PpIX. The other is to prevent residual yellow and red light from the LED to enter the bladder as this would confound the autofluorescence of the bladder mucosa and the sensitised red fluorescence from the tumours.

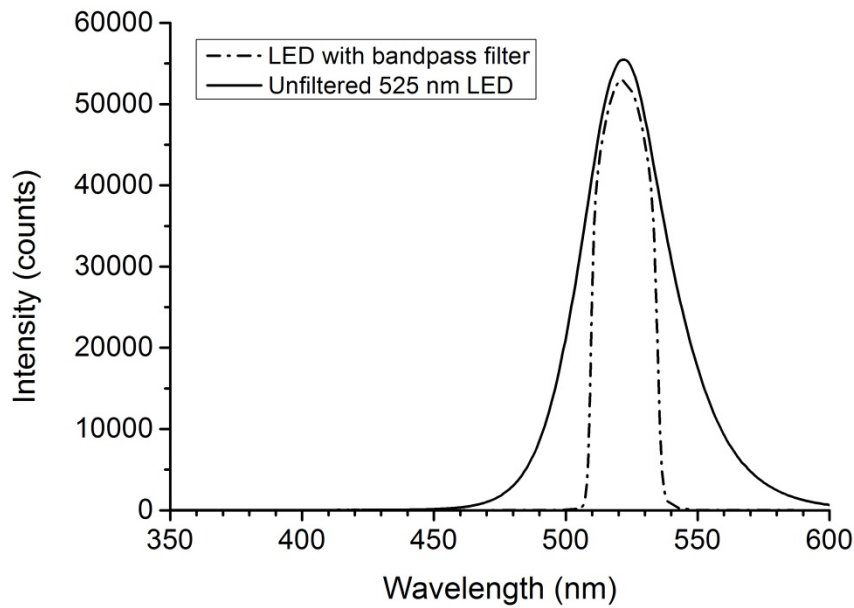


Figure 2. The emission spectrum of 525 nm LED PT-121-TE from Luminus Devices without bandpass filter and with a Semrock Brightline 525-25 filter with a bandwidth (FWHM) of 25 nm.

2.4 Spectrometers and power meter

The absorption spectrum of urine was recorded using a UV-2700 double-beam spectrophotometer (Shimadzu, Holm & Halby, Denmark). The fluorescence spectra of PpIX and urine were measured using a Fluorolog 3 spectrofluorometer (Horiba Jobin Yvon, Longjumeau, France). PpIX samples prepared as described in section 2.1 were placed in a suitable fluorescence cuvette in the spectrofluorometer and measured in right-angle geometry. The spectral output of the high-power LED light source was measured using a QE65000 CCD spectrograph from Ocean Optics (Dunedin, FL, USA). The power density of the light source was measured using a PM100USB power meter fitted with a S120VC photodiode power sensor both from Thorlabs (Thorlabs AB, Gothenburg, Sweden). To prevent saturation of spectrometer and power meter, a reflective neutral density filter ND with an optical density of 4 was used from Thorlabs (Thorlabs AB, Gothenburg, Sweden).

2.5 Optical components used in the experiment

As this proof-of-principle is based on commercially available cystoscope instrumentation, no alterations can be made to the hardware. In particular, this means that the filter for blocking the reflected excitation light transmitted by the cystoscope from the bladder cavity cannot be incorporated in either the cystoscope (22 French cystoscope, Karl Storz, Tuttlingen, Germany) or the attached camera (Tricam Storz, Tuttlingen, Germany).

It has previously been reported (Gahlen *et al* 2000) how the use of a longpass optical colour filter in the eyepiece of the cystoscope is required to block the

excitation light of the cystoscope light source. The purpose of this filter is to enhance the sensitised fluorescence of the tumours and autofluorescence of the healthy tissue of the bladder mucosa.

Such filters come in two generically different versions. One is based on interference filter technology and is often referred to as an edge filter. The others are based on absorption colour filters such as those supplied by Schott and Hoya. It is our experience that a blocking filter based on a thin film interference coating used in the eyepiece of a cystoscope suffers a serious drawback: As the cystoscope images objects with a distance from the distal end of the cystoscope ranging from 1 to 10 cm, an interference based bandpass filter will have light entering at very differing angles. In the case of an interference filter this will cause a different bandpass over the aperture of the eyepiece. This is not desirable in a fluorescence imaging setup.

A colour filter based on absorption like a Schott or Hoya filter on the other hand would provide the imaging system with angular independent bandpass over the entire aperture when placed in the eyepiece of the cystoscope. In our experiments, a Hoya OG56 filter (diameter 12.5 mm and 2 mm thickness) was placed in the eyepiece of the cystoscope before the Storz Tricam was attached to the eyepiece. The Storz Tricam was operated in the PDD mode. In this mode exposure time of the CCD camera typically is set at 1/15 second.

3. Results and discussion

3.1 Spectral measurements of urine and Protoporphyrin IX

Urine exhibits strong fluorescence in the blue-green spectral range when excited with blue light of commercially available endoscopic systems for photodynamic diagnosis. In order to assess the wavelength range that will excite the metabolites of urine, the absorption spectrum of urine was recorded using a Cary100 spectrophotometer as shown in figure 3.

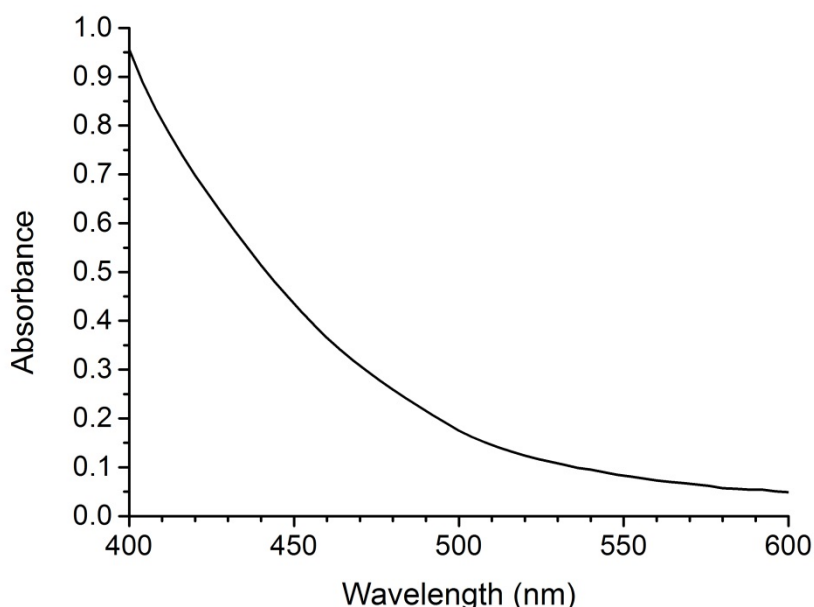


Figure 3. VIS Absorption spectrum of urine in a 10 mm disposable plastic cuvette measured in double beam UV 2700 spectrophotometer with a 10 mm OS cuvette containing MilliQ water in the reference beam.

It should be noted that the amplitude of the absorption spectrum of human urine varies from person to person. The spectral signature, however, is the same. This means that the spectrum shown in figure 3 is representative for people without unusual medical conditions like porphyria (Castrow *et al* 1968).

Figure 3 shows that light (430nm centre wavelength) of commercial available PDD systems, is strongly absorbed by urine (50% at 430 nm) and will cause the urine to fluoresce, but light with wavelengths above 500 nm are only absorbed marginally (10% at 525 nm) by urine in a 10 mm cuvette. It is, however, worth mentioning that the optical path length of the bladder is significantly larger than 10 mm due to fact that the urine is excited in backscattered fluorescence geometry rather than simple transmission geometry, the total optical path length more likely is 200 mm in the worst case and 50mm to 100 mm under clinical observation conditions. Assuming the absorbance to follow the Beer-Lambert law, further increasing the optical path length causes an even greater absorbance of the blue excitation light and hence a much stronger fluorescence signal from urine can be expected at 430 nm than 525 nm. The bladder can be regarded as a cuvette during cystoscopic procedures as it is inflated by water to allow a full view of its surface by the surgeon.

It should be noted that absorption by a metabolite does not necessarily lead to fluorescence *per se*. Based on the work of other groups (Lovisa *et al* 2010) and (Leiner *et al* 1987), however, similar absorption spectra have been recorded and correlated to the fluorescence of urine. Previously published methods for suppressing this green fluorescence have only reported results made *in vitro* (Lovisa *et al* 2010).

The results of the fluorescence measurements shown in figure 4 demonstrate a correlation between light absorption in urine and the magnitude of fluorescence of urine and PpIX respectively.

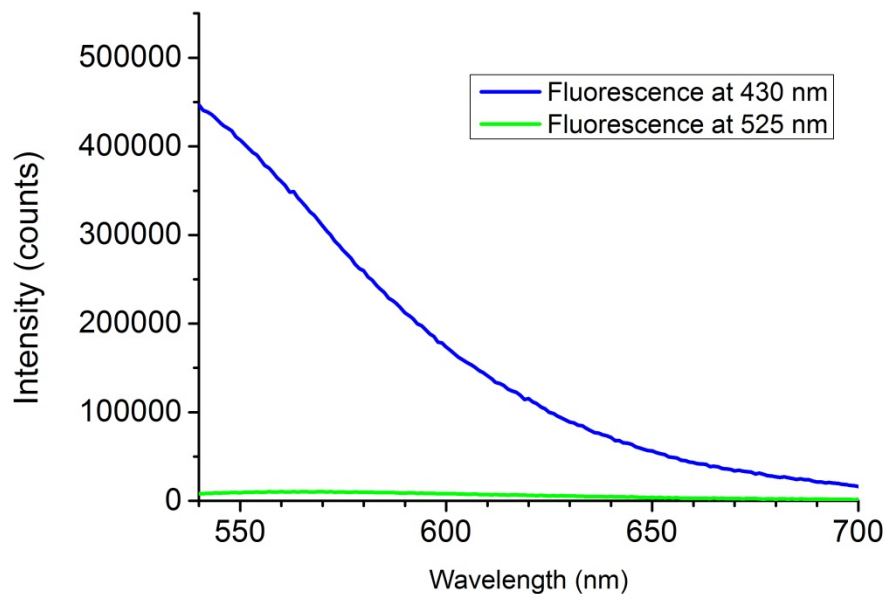


Figure 4. Fluorescence spectra of urine measured in right-angle geometry in disposable plastic fluorescence cuvettes with optical path length 10 mm measured at 430 nm and 525 nm excitation wavelengths respectively.

It is, however, important to note that fluorescence spectra shown in figure 4 only reflect the spectral properties of urine *in vitro*. During PDD in a bladder, the intensity of green fluorescence of urine will completely confound that of PpIX as the path length of excited volume is more than an order of magnitude larger than that of the PpIX containing tumour.

It can be inferred from figure 4 that 525 nm is beneficial for reducing the fluorescence of urine. The question, however, is whether it will be suitable for fluorescence excitation of PpIX?

The spectrum recorded by a Shimadzu UV-2700 spectrophotometer shown in figure 5 reveals that PpIX possesses minor absorption peaks located in a spectral region where the spectral absorption of urine, as shown in figure 3, is known to be very small.

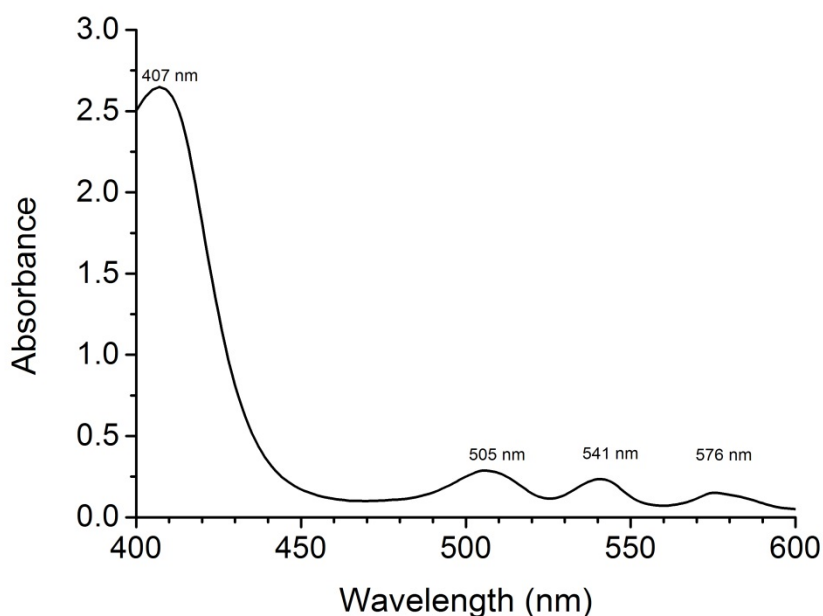


Figure 5. Absorption spectrum of 100 μ M Protoporphyrin IX di-sodium salt in PBS with 1% Triton X-100 recorded in OS cuvette with a path length of 10 mm and OS cuvette with a path length of 10 mm containing MilliQ water for reference. The absorption peaks have been located using the peak finder function of the UV-2700 spectrophotometer software.

It could be argued that the decrease in absorption would lead to reduced fluorescence from PpIX. As the fluorescence spectrum of PpIX shows in figure 6 this appears not to be the case. The spectra also demonstrate how the fluorescence of urine can be reduced by more than an order of magnitude using a longer wavelength for excitation of PpIX. As can be observed from the fluorescence spectrum of a 100 μ M di-sodium PpIX PBS solution with 1% Triton X-100

shown in figure 6, the fluorescence intensity of PpIX is not compromised by changing the excitation wavelength from 430 nm to 525 nm. An observation substantiated by the cystoscope picture in figure 7a, where the bright red fluorescence of bladder tumours can be observed clearly *in vivo* using the green LED light source developed in our laboratory.

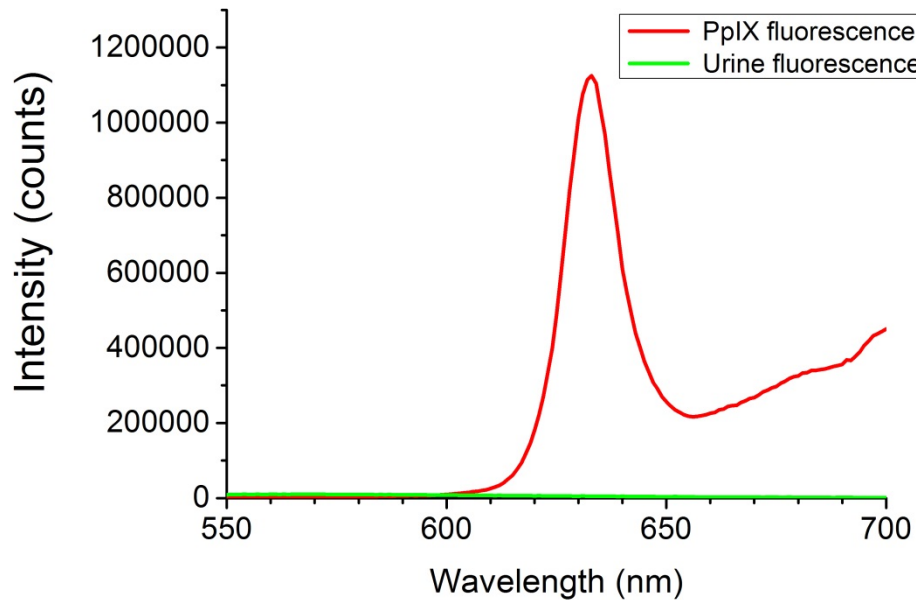


Figure 6. Fluorescence spectra of a 100 μ M PpIX-disodium salt in PBS with 1% Triton X-100 and urine at 525 nm excitation measured in disposable plastic fluorescence cuvettes with 10 mm path length.

3.3 *In vivo* test of 525 nm excitation light for PDD of bladder tumours

Based on the observations that the blue light from commercial PDD light sources cause significant green fluorescence in the bladder during PDD, a proof-of-principle cystoscope set-up based on the green LED light source described in section 2.3 was implemented.

For a proof-of-principle, 2 patients were selected for testing the efficacy of replacing the conventional light source in the OR and one patient in OPD by the new LED based light source emitting light at 525 nm. The patients were treated in accord to the Helsinki Declaration, the regulations of the local ethical committee, the Danish guide lines for treatment of bladder cancer, and had given informed consent before surgery. The patient in the OR had previously had a bladder tumour identified during endoscopy performed in the outpatient department. A Karl Storz PDD D-light system and Karl Storz endoscope for white light (not for blue light) was used (22 French cystoscope, Karl Storz, Tuttlingen, Germany) was used for the procedures in the OR. The patient in OPD was admitted for a follow-up on a TUR-B.

In the beginning of the surgical procedure the cystoscope was introduced to the bladder in white light and the bladder examined in white light. Then the white

light source was replaced with our 525 nm LED light source, and a Hoya OG56 longpass filter was placed between the eyepiece of the cystoscope optics and the CCD-camera to allow observation of PpIX fluorescence from the tumours and the autofluorescence from the healthy bladder tissue. Fluorescence from the tumour was recorded at the same time as urine was observed in the bladder but without green colouring (figure 7a). Furthermore, it was found that a clear picture could be obtained of the bladder mucosa using only autofluorescence as shown in figure 7b. It is noteworthy that the ostium is also visible in figure 1, before the bladder is filled with urine. During observation of this region in the cystoscope, using the LED light source at 525 nm, it could be observed that urine was flowing out of the ureter as the flow caused optical turbulence similar to that of a hot spring, but no green fluorescence due to the change in excitation wavelength.

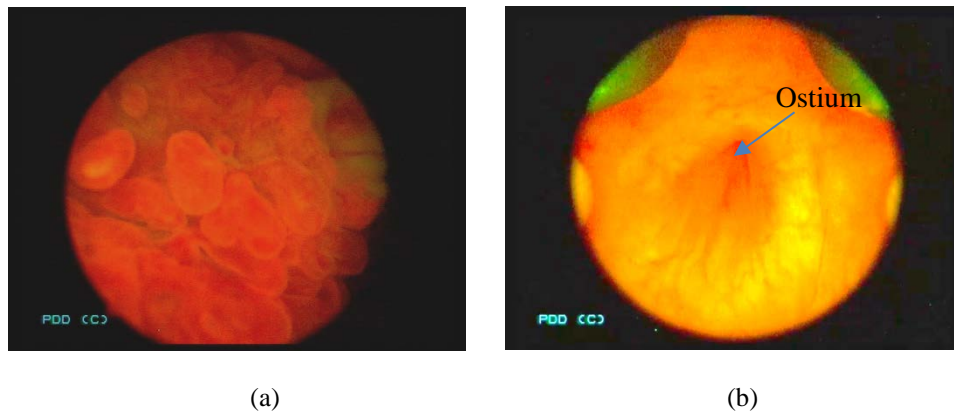


Figure 7a and 7b. The pictures show that the LED light source presented in this article is capable of exciting both PpIX sensitised fluorescence of tumours (7a) in a bladder as well as autofluorescence of the bladder mucosa (7b) with urine present inside the bladder, without changing the wavelength of the cystoscope light source as would be the case in conventional systems for PDD of tumours in the bladder. In 7a the orange-red fluorescence caused by PpIX in the egg-shaped tumours with a size of 3-5 mm can be seen, covering most of the field of vision. The arrow in figure 7b points to healthy bladder mucosa with yellow autofluorescence. Both images recorded using a Storz rigid cystoscope with Storz Tricam® and Hoya OG56 filter in the eyepiece. © Gregers G. Hermann.

It is important to realise that the use of the new LED-based light source enables the surgeon to observe the red fluorescence from tumours as well as autofluorescence from healthy tissue, which is typically yellow with a slight green tint caused by a controlled leaking of excitation light through the rejection filter in the eyepiece of the cystoscope optics. Contrary to normal PDD procedures where the surgeon switches between two settings (blue light and white light), only one setting is required with the new 525 nm LED light source.

To make a comparison between white light illumination of the bladder and autofluorescence (AF) imaging based on excitation with the 525 nm LED light source, two videos were recorded with the same patient. As can be seen in figure 8 the image is still sharp, although in a different colour due to AF. The cauterizing probe in the picture indicates that surgery could be carried out using AF as the only illumination source.

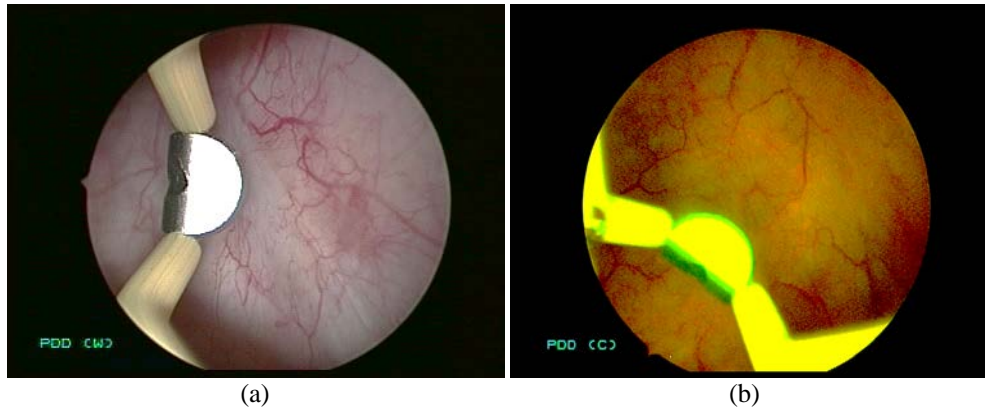


Figure 8a and 8b. The pictures show a part of a healthy bladder mucosa in conventional white light cystoscopy (8a) and using our green LED light source for exciting autofluorescence of the healthy bladder mucosa (8b). The use of a single green LED light source facilitates observation of the entire bladder mucosa using autofluorescence and tumours by sensitised fluorescence concomitantly. The surgeon will not have to switch between two lamp settings. The probe seen in both pictures is an electrically heated probe for cauterizing tumours. Both images were recorded using a Storz rigid cystoscope with Storz Tricam® and Hoya OG56 filter in the eyepiece. © Gregers G. Hermann.

The last trial performed was to test the hypothesis, that the LED light source could be used in a flexible cystoscope. The LED light source was tested with a patient who had been referred to the OPD for a follow-up cystoscopy.

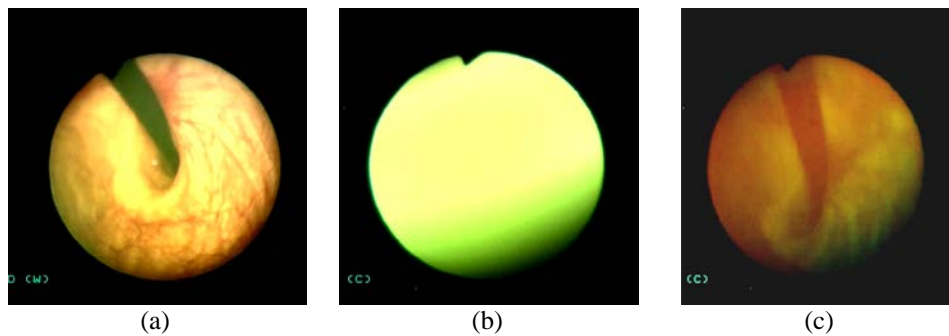


Figure 9. The sequence of images shows (a) white light observation of bladder wall and flexible cystoscope optics (black tube), (b) the same scene now with blue light (430 nm) illumination for photodynamic diagnosis with distinct green urine fluorescence, and (c) the same scene with green LED illumination of the bladder. Note the absence of green fluorescence from urine that otherwise obscures the image as shown in 9b. All images were recorded using a Storz flexible cystoscope with Storz Tricam® and Hoya OG56 filter in the eyepiece © Gregers G. Hermann.

The images shown in figure 9 demonstrate that the proposed concept of using green light in flexible cystoscopes in OPD is viable. The images obtained with the flexible cystoscope, however, were not of the same quality as the ones obtained by a rigid cystoscope. The quality, though, merely reflects that a good quality

flexible cystoscope was not available on site at that time in the outpatient department.

4. Conclusions

We have shown *in vivo* that the ubiquitous problem of green fluorescence during PDD for bladder tumours, particularly when using flexible cystoscopes, can be circumvented by selecting excitation light in the green spectral range rather than blue spectral range currently used by the commercial systems. It is noteworthy that the light source presented in this article is used with standard white-light cystoscopes and light guides, without any blue-light optics.

Furthermore, it was also observed that using green excitation light also made it possible to use the autofluorescence of the mucous membrane in the bladder for general observation, provided that a suitable bandpass filter was placed in eyepiece of the endoscope optics. This may possibly replace the use of white light sources for general viewing during cystoscope based PDD procedures for removal of bladder tumours.

It is important to emphasize, that the results presented in this paper are to be regarded as a proof-of-principle. Based on the observations reported in this paper, a series of experiments will be needed to prove the efficacy of the new method in order to establish a proper protocol for this method in the clinic, including a study of a larger cohort of patients. This will provide us with better statistical grounds for substantiating the advantages of the new method compared to conventional PDD with two illumination modes, i.e., blue for fluorescence excitation and white light for general observation. Another very important issue to be addressed in the future, if the proposed technique is translated from proof of principle to a new medical device, is the regulatory environment. Approval of the device will be mandatory; in the EU via CE marking and in the US via FDA approval.

Acknowledgements

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